Innovative platform technologies for stabilization and controlled release of proteins from polymer depots

Stankovic, Milica

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Summary and concluding remarks

Milica Stanković

Summary

Recent developments in the area of biotechnology and molecular biology have resulted in an increased interest in the use of peptides and proteins for treatment of a variety of diseases and disorders. However, due to their instability in GI tract and poor permeability over the intestinal wall these macromolecules require parenteral administration and often frequent administrations because generally proteins exhibit a short half-life in the circulation. Polymer based injectable depot preparations for the sustained release of proteins over prolonged periods of time offer an attractive alternative to frequent injections, because amongst other reasons they increase patient compliance and reduce therapy costs.

Hot melt extrusion (HME) is a technology extensively used to prepare polymer based implants for the controlled release of drugs. This method offers numerous advantages over the traditional emulsification based microencapsulation methods. Nevertheless, the encapsulation of proteins into polymer matrices using this technique still presents a challenge due to highly sensitive nature of these macromolecules. Therefore, stabilization of proteins prior to, during and after encapsulation is of a high interest. Further, the use of biodegradable polymers, which are metabolized in the body into non-toxic products and subsequently excreted, is preferred, as they do not require surgical removal after the drug is released.

The aim of the studies described in this thesis was to investigate the mechanisms of stabilization of proteins by sugar glass technology, to encapsulate sugar glass stabilized proteins into innovative biodegradable polymer matrices by means of HME, and to study the release of the proteins from these matrices. The path this research followed consisted of two major steps:

1. Evaluation of the spray-drying technology for the stabilization of proteins using sugar glasses, followed by the incorporation of the stabilized proteins in biodegradable polymers.

2. Evaluation of novel multiblock copolymers as matrices for the controlled release of proteins. HME was chosen as a method for manufacturing of depot formulation with encapsulated proteins.

In **chapter 2**, a literature review is given on the HME technique and the polymers used. The application of HME for the preparation of different formulations intended for oral, sublingual, transdermal, intraocular and parenteral administration is evaluated. Biodegradable and non-biodegradable polymers that are widely used in this technique are described and the most relevant techniques to characterize polymer based HME products are summarized.

In **chapter 3,** the role of the vitrification and the water replacement mechanism in protein stabilization using sugar glasses has been studied. Alkaline phosphatase was used as a model protein. The protein was incorporated into a matrix of inulin and trehalose in the glassy state using spray-drying. Due to the glass forming ability of ammediol, it was not only used as a buffer but also as a plasticizer. Variation of the sugar/buffer mass ratio resulted in spray-dried products with different glass transition temperatures. The protein storage stability at different glass transition temperatures has been studied. It was shown that when the glass transition was below storage temperature, protein stabilization was controlled by the vitrification mechanism. However, when the glass transition temperature was above storage temperature, the water replacement mechanism was dominant.

In **chapter 4** the synthesis and characterization of a novel hydrophilic multiblock copolymer composed of the two phase-separated blocks is described. One block consisted of semi-crystalline poly(ε-caprolactone) (PCL) (30 wt% of the copolymer) and the other consisted of the amorphous poly(ethylene glycol)–poly(ε-caprolactone) block [PCL-PEG] (70 wt% of the copolymer). A major advantage of this copolymer was that it could be extruded at a much lower temperature (55 °C) than the widely used poly(lactide-co-glycolide) (>85 °C). Furthermore, it was studied whether the model protein lysozyme could be encapsulated into the polymer by HME, without compromising its biological activity.

An aqueous solution of lysozyme was first spray dried with and without inulin to obtain powder with a lysozyme/inulin weight ratio of 1/0, 1/1, 1/2, 1/3. The spray-dried powders had a similar particle size distribution (X_{50} was 1.09 - 1.50 μm). Subsequently, the powders were encapsulated into the polymer by HME resulting in 10 wt%, 20 wt%, 30 wt% and 40 wt% of lysozyme/inulin load, while keeping the lysozyme load the same. Furthermore, to evaluate the effect of particle size, lysozyme as received (X_{50} was 20.82 μm) was also encapsulated into the polymer. In all cases the enzymatic activity was fully maintained during HME. The *in-vitro* lysozyme release kinetics were evaluated, together with the enzymatic activity of the protein during 260 days of incubation in phosphate buffer (pH 7.4) at 37°C. It was observed that co-incorporation of different amounts of inulin resulted in an increased lysozyme release rate, indicating that inulin acted as a pore-forming excipient. The samples with a 10 and 20 wt% protein/sugar load released, after an initial burst, the protein continuously during 260 days with kinetics close to first order kinetics suggesting that after the burst release, at low loadings the release is governed by the protein diffusion through the polymer matrix. At higher loading, lysozyme release was very fast indicating that the lysozyme/inulin particles were connected via a percolating network, forming channels through which the protein diffused out. It was also observed that the initial release of lysozyme was highly dependent on the

protein particle size. The formulation consisting of 10 wt% spray dried lysozyme exhibited a smaller burst release than the formulation containing the same amount of lysozyme with larger particle size ("as received" material). After 70 days of the release, the release rate of protein from both formulations was similar, indicating that in this later stage, the release is not dependent on the particle size. Lysozyme remained 80 % active after the 180 days of release. It was also observed by scanning electron microscopy that the polymer morphology remained intact after 180 days of release, indicating slow degradation of the polymer. In conclusion, in this study it was shown that not only the protein/sugar load, but also the particle size of the protein should be considered when designing a controlled release device.

In **chapter 5**, the degradation kinetics of the multiblock copolymer described in chapter 4 was evaluated. It was further studied whether the degradation rate of this copolymer could be enhanced by increasing the amorphous (PCL- PEG) content of the polymer. Therefore, polymers with different x[PCL-PEG_{1500}]/y[PCL] block ratios (x/y being 30/70, 50/50, 70/30 (w/w)) were synthesized and characterized and the in-vitro degradation behavior of polymer-only implants was evaluated during 140 days of incubation in phosphate buffer (pH 7.4) at 37 °C. It was observed that the degradation rate of these copolymers increased upon increasing the [PCL-PEG]/[PCL] ratio. Additionally, the effect of molecular weight of the protein and the block ratio of the polymer on the release kinetics was investigated. Because the results of the studies described in chapter 4 clearly indicated that both protein particle size and protein loading influenced the release kinetics, we spray-dried proteins of different molecular weight (goserelin (1.2 kDa), insulin (5.8 kDa), lysozyme (14 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa)) with inulin in the constant weight ratio (1/10) to obtain uniform particle size distribution after which these powders were encapsulated in the polymers at constant powder (protein+inulin) load (11 wt%). All formulations were extruded at low temperatures (50 – 55 °C) and it was found that the structural integrity of proteins was fully preserved during extrusion. Furthermore, it was shown that the protein release rate increased with decreasing molecular weight of the protein and increasing [PCL- PEG]/[PCL] ratio of the polymer. It was concluded that small molecular weight proteins were released mainly by diffusion, but when the protein was larger than the polymer mesh size both diffusion of protein and degradation of the polymer played a role in protein release. Therefore, this study demonstrated that knowledge of both protein molecular weight and [PCL- PEG]/[PCL] ratio of the polymer are essential in designing the optimal delivery depot with tailored protein release.

In **chapter 6,** novel multiblock copolymers composed of semi-crystalline poly(ε-caprolactone) (PCL) blocks and amorphous blocks consisting of poly(D,L-lactide) (PDLA) and poly(ethylene glycol) (PEG) [PDLA-PEG] were synthesized.

The block ratio of these [PDLA-PEG]-*b*-[PCL] multi block copolymers was varied and the degradation of implants prepared thereof by HME was compared with implants prepared of 30[PCL-PEG_{1500}]-70[PCL], a copolymer which has been described in previous chapters. Furthermore, the release of the model proteins lysozyme and bovine serum albumin incorporated into polymer implants was studied.

The copolymers composed of [PDLA-PEG]-*b*-[PCL] showed increased degradation rate in the initial phase, which could be controlled by varying [PDLA-PEG]/[PCL] block ratio. As expected, it was found that with increased polymer degradation rate the protein release rate also increased. However, protein release from [PDLA-PEG]-*b*-[PCL] copolymers was incomplete, in contrast to [PCL-PEG]-*b*-[PCL] copolymers that showed slow and continuous release. The incomplete protein release was attributed to either irreversible interaction between the proteins and the polymer degradation products or to the physical entrapment of the protein into the semi-crystalline PCL matrix. In contrast, protein release from the slowly degrading [PCL-PEG]-*b*-[PCL] polymer was continuous and complete during the entire study, suggesting the absence of protein-polymer interactions or irreversible protein entrapment for this type of copolymer.

Concluding remarks and perspectives

There are many emulsification based microencapsulation methods, which are used to entrap molecules in polymer matrices. HME is a solvent-free process gaining a lot of attention in development of a large variety of dosage forms. Next to the many advantages this method may offer (discussed in the **chapter 2**), it often requires high temperatures and shear stresses that can be detrimental for unstable drug substances like proteins. Therefore, in this thesis a combination of two strategies was evaluated to overcome protein degradation during encapsulation in polymer matrices by HME: 1) pre-stabilization of the protein using sugar glass technology and 2) application of novel polymers that can be extruded at relatively low temperatures.

In the literature, the disaccharide trehalose is often referred to as the gold standard for protein stabilization during freeze- or spray-drying processes and subsequent storage. The oligosaccharide inulin is also known to be an excellent protein stabilizer [1]. We have, therefore, evaluated and compared the stabilizing capacities of trehalose and inulin (**chapter 3**). Although protein stabilization has often been ascribed to occur either through vitrification or through water replacement, we have shown that the difference between glass transition temperature (T_g) and storage temperature determines the mechanism of protein stabilization. When the T_g of the spray-dried products was adjusted to the same value by using ammediol

as a plasticizer, trehalose performed slightly better as stabilizer than inulin did. However, because pure inulin has a higher T_g than pure trehalose, inulin could be an attractive alternative to trehalose, especially when samples are stored at high relative humidities and temperatures. Additionally, the higher T_g of inulin would provide better stabilization of proteins during high temperature processes like HME.

The novel, hydrophilic multiblock copolymer based on PCL and PEG described in **chapter 4** showed favorable properties to be used as matrix for the encapsulation of proteins by HME. This polymer allowed for successful extrusion at 55°C, which is 30°C lower than the extrusion temperature required for PLGA. This low temperature could be beneficial for the encapsulation of thermolabile drug substances like proteins and peptides. Furthermore, the model protein lysozyme showed continuous release kinetics as compared to the biphasic release usually observed with PLGA. The lysozyme release was driven mainly by diffusion, while the polymer showed very limited degradation during the period the release occurred (260 days), which was further described in **chapter 5.** The slow degrading polymers could be favorable when molecules with a required release longer than 1 year are to be encapsulated [2–4]. However, for molecules below a certain hydrodynamic diameter, the release occurs faster than the polymer degradation rate [5]. Therefore, it might seem to be beneficial to develop a polymer with enhanced degradation kinetics, which will not exceed extensively the duration of protein release.

Faster degradation behavior could be obtained by replacing the [PCL-PEG] amorphous block in [PCL-PEG]-[PCL] by [PDLA-PEG]. However, we have shown that proteins irreversibly interact with degradation products of the [PDLA-PEG]-[PCL] polymer (**chapter 6**). Protein-polymer interactions have been previously described as an important cause of *in vitro* incomplete protein release from PLGA based depot systems. They have been ascribed to protein aggregation, protein adsorption to hydrophobic PLGA [6–10], electrostatic interactions between protein and polymer [7,11] and chemical modifications of protein, e.g. acylation [12–14]. We speculated whether the formation of polymer degradation products could be responsible for the protein-polymer interaction or whether the protein remained entrapped in the semi-crystalline block upon the dissolution/degradation of the amorphous block. However, the exact mechanism of this incomplete release should further be investigated in more detail. One of the possibilities to further evaluate the nature of the covalent protein-polymer bonding could be the analysis of samples by LC-MS after incubation with an aqueous NaOH solution. These methods would allow determination of additional peaks and identification of the molecular weights of the obtained compounds. Additionally, protein labeling and subsequent visualization after extrusion and during the *in-vitro* release could provide more information on the protein distribution inside the implants.

Experiencing incomplete release of protein as a possible consequence of polymer degradation, one can thus argue whether the polymer degradation should occur in parallel to protein release or whether massive polymer degradation should occur only after the entire protein has already been released to avoid the risks of these irreversible interactions. In the study described in **chapter 5** it was observed that certain degradation (or surface erosion) of polymer and formation of interconnecting channels was necessary for release of larger proteins. However, to avoid the risk of the interaction of proteins with the degrading polymer, ideally protein should be released from the polymer depot by diffusion. The diffusion of the protein from the polymer bulk could be enhanced by co-incorporation of pore formers into polymer matrix. This can be for example achieved by incorporation of PEG into the polymer matrix, thereby increasing the swellability of polymers (**chapter 5 and 6**) or by co-encapsulation of inulin as a pore-forming excipient (**chapter 4**).

Although we have shown that inulin sufficiently stabilized the model proteins lysozyme and albumin during the extrusion process, these proteins may be inherently much more stable than many other therapeutically relevant proteins e.g. interleukins, human growth hormone or TNF-Related Apoptosis Inducing Ligand. Additionally, other model proteins used in our study, i.e. goserelin, insulin and carbonic anhydrase, were extruded only in presence of inulin, hence their stability during the extrusion process without stabilizer remains questionable. Therefore, future investigations should be directed towards the evaluation of the therapeutically relevant thermolabile proteins and the capability of inulin to protect them during this process. Alternatively, when polymers with fusion temperatures much higher than 55 °C are required for the preparation of protein-loaded polymer depots, inulin could offer sufficient protection during the HME process. Future studies on this issue are therefore also recommended.

Since protein stability is of great importance during the encapsulation and controlled release from drug delivery systems, the use of *in-line* techniques for protein characterization (FTIR, CD, Raman) during the extrusion process or during the protein release and characterization would be of high interest to address the protein's structural integrity. Additionally, even though we have shown in this thesis that inulin preserved the proteins integrity during extrusion, it remains questionable to which extent the activity of inulin-stabilized proteins would be preserved under *in-vivo* conditions. Upon the administration of the implants and upon contact with the aqueous medium, the inulin particles will be wetted and will (partially) be dissolved before all the incorporated protein is released. Therefore, rational *in-vivo* studies using appropriate animal models would be required in order to give an answer to these questions. Moreover, the *in-vivo* pharmacokinetics studies should be performed to confirm the predictive value of *in-vitro* release profiles.

For the rational development of the protein loaded polymer matrices and optimization of protein stability, one must first determine the stage at which protein degradation occurs and identify the stress factors which compromise protein stability. Afterwards a rational stabilization approach can be followed to ensure the safety and efficacy of the protein-loaded polymer depots. These developments could result in the clinical availability of sustained protein release systems.

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